(Title of Thesis)

Studies on Endamoeba Histolytica, Its Accompanying Bacteria, and Blastocystis Hominis.

by

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STUDIES ON ENDAMOEBA HISTOLYTICA, ITS ACCOMPANY-BACTERIA AND BLASTOCYSTIS HOMINIS.

INTRODUCTION:

During the time that Endamoeba histolytica has been cultured in the laboratories of the University of Kansas, certain problems concerning its growth have arisen. Periods of irregular growth have been noted by everyone who handled them. At times, for apparently no reason, there would be a sudden decrease in numbers of the amoeba in the cultures after their usual transfer and incubation. This condition of feeble growth would last for several transfers, and then there would be a rapid improvement which would prevail for a time. Since these two conditions, that of abundant growth and that of feeble growth were continually occurring, a periodicity was suggested, and it was to study this phemomenon that certain of the experiments which follow were carried out. It was thought that bacteria might play a part in this variability, therefore a cultural study of the bacteria isolated from E. histolytica cultures and an investigation of the general procedure which was being used in culturing the amoeba were undertakem.

Since E. histolytica together with its accompanying bacteria has been used extensively in making antigen for complement-fixation work, it was thought that it might be advantageous to rid the amoeba of as many bacteria as possible, hence the study of the effects of certain chemical and physical factors on the cysts of E. histolytica was undertaken.

The last section of this work has to do with a series of complement-fixation tests with E. histolytica and Blastocystis hominis. The latter organism was grown from the stools of three people with no apparent amebiasis who gave a four plus complement-fixation reaction using Sherwood's alcoholic lipoid antigen. It was thought possible that there might be some fixation caused by B. hominis as a comtaminate in the cultures of E. histolytica from which the antigen had been made. Tests with normal sera which fixed complement and with that which did not fix complement with Sherwood's alcoholic lipoid antigen were planned to compare the antigens prepared by different methods in this work. For these reasons, then, the antigenic studies of E. histolytica and Blastocystis hominis were undertaken.

REVIEW OF LITERATURE:

A great many attempts have been made for many years to cultivate Endamoeba histolytica on artificial media, but it is only in recent years that the attempts have been sucessful. All of the early work, prior to 1925, where cultivation has been reported, is debatable. It has been thought that perhaps the workers have cultivated some other organism than E. histolytica.

Cutler in 1918, (1) reported two media; one with emulsified egg, with or without the addition of blood; the other was a boiled extract of human blood clot, to which was added peptone. His work has never been sucessfully repeated and the sucess of these media is debatable.

Boeck and Drbohlav, 1925 (2), announced a medium for the cultivation of E. histolytica and these claims have been substantiated by many workers since. Boeck and Drbohlav referred to their medium as the Locke-egg-serum medium. It was made as follows:

1. Four eggs, washed with alcohol were broken into sterile flask containing glass beads.

50 c.c. of Locke's solution was added.

2. Test tubes were filled with enough to make a one to one and onehalf inch slant on coagulation by heat.

3. The slants were then placed in an inspissator and heated to 70 degrees C. until solidified. They were then transferred to an autoclave and sterilized for 20 minutes at 15 pounds pressure.

4. Tubes were covered to a depth of one cm. above the slant with a mixture of 8 parts sterile Locke's s solution and one part sterile inactivated human blood serum. Incubate for sterility.

5. Locke's solution:

Dist. water	1000.00 c.c.
NaCl	9.00 gm.
CaCl	.20 gms.
KCl	.40 gms.
$NaHCO_3$	.20 gms.
Glucose	2.50 gms.

This solution may be sterilized in the Arnold or in an autoclave.

Boeck and Drobahlav brought out in this paper many things of zoological interest comparing the morphology of the ameba as found in the animal

body with those grown in culture. They found that ameba in the absence of red blood cells andtissue juices would engulf bacteria. The ameba, however, perfer the absorption of soluable products of tissue cells. The ameba were unable to encyst in this medium although they proved pathogenic for kittens.

In 1926, Craig(10) proposed a liquid medium composed of Locke's solution and serum. In a later communication(11) he purposes a further modification with Ringer's solution and human blood serum, or normal salt solution and human blood serum. His technique of cultivation has been tried by other workers experimentally without sucess.

Dobell and Laidlaw, 1926 (3), introduced sterile rice flour to the coagulated egg medium of Boeck and Drobahlav causing the ameba to encyst. They found that cysts required carbohydrates. Since the more complex sugars are not readily split by many bacteria, the rice flour furnished a carbohydrate available only for the ameba. Starch splitting bacteria were eradicated by the addition of 1-20,000 dilution of acriflavine. The ameba grow in the presence of this dilution of acriflavine readily and the starch splitters among the bacteria are sucessfully killed. A method of ridding the cultures partially of bacteria was also introduced.

The material was concentrated by centrifugation on sucessive days. It was then allowed to stand in a 0.2% solution of HCl for two hours. After being neutralized with a weak solution of sodium bicarbonate with neutral red as an indicator, it was centrifuged again and inoculated into sterile media.

Cleveland and Sanders, 1930 (4), introduced the medium which is now used sucessfully in the laboratories of the University of Kansas. The medium consists of a liver infusion agar slant covered with a sterile serum-saline solution to which sterile rice starch has been added. The instructions for making this medium are given more in detail ~~are given~~ under technique in the first section of this work. The remainder of the report by Cleveland and Sanders was principally of zoological interest, consisting of the study of a pure line strain, with the establishment of some different forms previously identified by other workers under different names, as all being forms of E. histolytica. Cleveland announced that encystation depended on three things, rapid growth in twenty-four hours, presence of rice flour, and the presence of certain kinds of bacteria. He also thinks that the liver medium may be a factor in encystation.

Cleveland and Sanders, 1930 (5), tried

dyes and chemicals without success in freeing cysts of bacteria. No details of this work were given. However bacteria free amoeba were obtained by direct liver inoculation. Five tenths of a cubic centimeter of attenuated organism were inoculated directly into the liver of a cat following a laparotomy. About seven to nine days were allowed to elapse; the cat was etherized; the amoeba abscess was removed under aseptic conditions, cut in small pieces, and inoculated into liver medium. In no instance did the bacteria free amoeba live longer than four days. However it was possible to obtain cultures of amoeba growing with certain pure cultures of bacteria. This was done by inoculation into liver medium which contained a loopful of a pure culture of bacteria, from the bacteria free amebic abscess.

Cleveland and Collier, 1930 (6), gave in detail the technique for culturing E. histolytica. This is described in Section I under technique.

Cleveland and Sanders, 1930 (7), made some studies on the virulence of E. histolytica using a strain which had been kept on laboratory media for varying lengths of time. These workers found that after cultivation of a year, the amoeba lost almost entirely the ability to maintain themselves

in the liver and in the intestine in vivo. After liver passage, infectivity was increased from twenty to seventy-five percent Their findings point to the important part bacteria play in infection, since amoeba inoculated into liver with fifth passage bacteria(i.e. those bacteria which had been passed through the livers of five cats without amoeba) proved to be highly infective; whereas, when the amoeba were inoculated with freshly isolated bacteria, there was no infection. They failed to demonstrate intestional amoebiasis except when the organisms or the bacteria with the organisms had been passed through cats.

Kagy and Faust, 1930 (12), following the work of Cleveland and Sanders and on the suggestion of the latter workers that something in the dehydrated liver caused encystation, fed dogs infected with E. histolytica raw liver, to study the effect of raw liver in the large intestine. The effect seemed to be the formation of more cysts. before the feeding of liver, trophozoites were found in large numbers in the feces; following the feeding, the cysts were materially increased. These workers also report a complete loss of the ability to form tropho-

zooites after being placed in Cleveland's medium.

Very little work has been done on the effect of dyes and chemicals on E. histolytica. Mention has already been made of Cleveland and Sanders in which no detail as to technique was given. Brown, 1930 (13), reports on the effect of vital dyes on E. histolytica. Neutral red up to a one percent solution was non toxic and stained the protoplasm distinctly, the amoeba remaining motile for some time. Janus green was also non toxic up to one percent, but stained more slowly than neutral red. Brilliant cresyl blue was unsatisfactory. Trypan blue and Nile blue were non toxic up to 0.5%. Neutral red and Janus green proved most satisfactory.

STUDIES ON ENDAMOEBA HISTOLYTICA, ITS ACCOMPANY-
BACTERIA AND BLASTOCYSTIS HOMINIS.

SCOPE OF WORK:

The scope of work may be summarized as follows:

1. The study of the growth of E. histolytica in Cleveland's medium.

2. The study of bacteria isolated from amoeba cultures.
   a. The study of some color reactions in isolated strains of Pseudomonas.

3. The study of the effects of certain chemical and physical factors on the cysts of E. histolytica.

4. A brief study of some complement-fixation tests with E. histolytica and Blastocystis hominis.

I. THE STUDY OF THE GROWTH OF ENDAMOEBA HISTOLYTICA IN CLEVELAND'S MEDIUM.

PURPOSES:

1. To increase numbers of amoeba by improving the technique of transfer.

2. To study the number of amoeba in cultures at different periods of growth, to ascertain the optimum time to transfer.

3. To study the causes of a sudden decrease in numbers at certain periods.

AMOEBA USED:

Two strains of E. histolytica were used in these experiments. They were called "s" and "w" strains. Both were obtained by the Bacteriology Department of the University of Kansas from Miss Bertha Kaplan of the Department of Hygiene, the University of Chicago, Chicago, Illinois. Both strains have been carried in this laboratory for about two years

TECHNIQUE:

Medium: The medium used in this work with E. histolytica, is that devised by Cleveland and Sanders (4), and prepared in accordance with their directions. Thirty grams of Difco Liver Infusion

agar, especially prepared by the Digestive Ferments Company of Detroit, Michigan, (500 gms. beef liver, 10 gms. peptone, 5gms. NaCl, 20 gms. agar.) is dissolved in 1000 cc. distilled water, by heating to boiling temperature. This is tubed in about five to seven cubic centimeter lots, autoclaved at fifteen pounds pressure for twenty minutes, slanted and cooled. These slants are kept at 20 degrees Centigrade until used. Before inoculation, the slants are covered with a 1:6 serum-saline solution and a loopful of sterile rice flour is added. The serum-saline solution is composed of one part freshly inactivated horse serum, sterilized by Berkefeld filtration, and five parts, sterile physiological salt solution. This is usually made up in lots of 240 cc. (200cc. saline to 40 cc. horse serum) and kept in the ice box at six to eight degrees Centigrade until used.

The rice starch or rice flour is prepared as follows: The rice starch is placed in three to four cc. lots in 15cc. glass tubes and heated in the hot air oven at 180 degrees C. for one hour. It should not be allowed to get any hotter than this, as the starch may be broken down to a soluble form, which makes it available for the carbohydrate fermenting bacteria and a subsequent change in the reaction of the medium may follow.[1] A

three millimeter loopful of this is added to each tube.

The tubes with the serum-saline and rice starch were incubated at 37 degrees C. for twenty-four hours for sterility. Only sterile tubes should be used, as it was found that sterility is an important factor in keeping the cultures growing abundantly.

Method of transfer: The technique of Cleveland and Collier(6) was closely followed. The amoeba were grown for varying lengths of time. They wereusually transferred after 48 hours growth at 37 degrees C. An ordinary one cc. sterile pipette was used. About three or four drops of sediment was transferred to a fresh tube. Care was taken to transfer as little as possible of the liquid with the sediment, as it contained many bacteria, and few amoeba. Four cultures of each strain were kept regularly as stock, each culture transferred with a separate sterile pipette. Before inoculation, the tubes containing the sterile media, were immersed in warm water, approximately 37degrees Centigrade.

Method of counting: The amoeba were counted on a ruled slide such as is used in counting leukocytes. A drop of the undiluted culture was used. Four areas representing four square

millimeters were counted, and the amount in one cubic millimeter calculated by multiplying the average by ten.

EXPERIMENTAL:

When the amoeba were first taken over, they they had been transferred for only a short time on the Cleveland medium. For over a year they had been grown on the Boeck- Drbohlav medium (2), and had not become adapted to Cleveland's medium, so that they would grow in as great numbers as he described. It was thought that some improvement on the technique of transfer might be made so that the amoeba would multiply more rapidly. Therefore, fields were counted daily on twenty-four hour cultures which were repeatedly transferred at twenty-four hours, and on forty-eight hour cultures which were repeatedly transferred at forty-eight hours; likewise counts were made on seventy-two and ninety-six hour cultures. This was continued for three months, until under treatment, the amoeba became so numerous as to be impractical to keep up the work. Table I shows a representative succession of counts made on cultures repeatedly transferred at twenty-four hours.

Washing the amoeba was tried with considerable success in increasing their numbers. The sediment from two twenty-four hour cultures was placed in a sterile centrifuge tube, washed twice with sterile physiological

saline, running the centrifuge at its lowest speed, about 850 revolutions a minute, to prevent injury to the amoeba, and to remove as many bacteria as possible in the supernatent fluid.

The washed sediment was transferred to a sterile tube of Cleveland's medium. Considerable increase redulted in the succeeding forty-eight hours. Whenever the cultures appeared to be growing weaker, washing in sterile saline seemed to improve growth.

Cleveland's technique,(6), as given above, was followed carefully with special attention to these three things: Placing only one loopful of rice flour in the medium; transferring only the sediment and no liquid; observing strictly aseptic conditions. Under these conditions, immediate improvement followed.

From the daily counting experiments, very little was learned. Those cultures which were transferred at forty-eight hours(Table II) seemed to maintain their numbers somewhat better than those than those transferred at twenty-four or at seventy-two hours(Tables I and III). Although frequently the numbers showed an increase at seventy-two hours, successive transfers at this interval of incubation seemed to result in steadily decreasing numbers(Table III). The same may be said for the twenty-four hour

growths. The ninety- six hour cultures were unsucessful. No further information was gained by counting the amoeba on sucessive days from the same culture(TableIV). Therefore forty-eight hours of growth, as recommended by Cleveland, was adopted as the best time for regular transfer.

Since the culture was first obtained, there has been observed a sudden unexplainable decrease in numbers which would last over a period of a few days, and then a return to the usual number was observed. A cycle was first suspected, and it was thought that the daily counting would reveal a regularity. Since the tubes were transferred each with a separate pipette, the cycle if there were one, should appear in all the tubes at onee. This necessitated, a careful tabulation of each tube inoculated. On January 4, February4, and April 2, the amoeba in all the cultures showed a sudden decrease. However no decrease was observed on the same dates in the tubes carried by other workers in the department, whose cultures were from the same stock. No simultaneous decrease in all the tubes has been observed since then. Frequently, the amoeba would decrease in one tube and be at their usual number in the other cultures. Therefore, it was thought that the decrease on these regular dates was a coincidence,

and that it was probably owing to contamination with change in the bacterial flora, which affected the reproduction of the amoeba, or to some other undetected error in technique.

SUMMARY:

1. Cleveland's technique,if followed closely, is sucessful in producing large numbers of amoeba.

2. The best time to transfer is at forty-eight hours.

3. Washing the amoeba with sterile saline seems to improve a culture which has been growing poorly.

4. A sudden decrease in numbers is probably due to comtamination or error in technique.

EXPLANATION OF FOLLOWING TABLES.

Table I shows the number of amoeba per cubic millimeter found in cultures transferred at twenty-four hours.

Table II shows the number of amoeba per cubic millimeter found in cultures transferred at forty-eight hours.

Table III tabulates the number of amoeba per cubic millimeter in cultures transferred at seventy-two hours.

Table IV tabulates the number of amoeba per cubic millimeter found in the same culture on successive days during which no fresh transfer has been made.

TABLE I

Counts made on E. histolytica cultures transferred repeatedly at 24 hours.

DATE	STRAIN	AGE	NUMBER PER CU.MM.
11-4-30	W	24 hrs.	22
11-5-30	"	" "	45
11-6-30	"	" "	29
11-7-30	"	" "	12
11-8-30	"	" "	24
11-9-30	"	" "	95
11-10-30	"	" "	50
11-11-30	"	" "	105
11-12-30	"	" "	40
11-13-30	"	" "	29
11-14-30	"	" "	50
11-15-30	"	" "	63
11-4-30	S	24 hrs.	27
11-5-30	"	" "	35
11-6-30	"	" "	45
11-7-30	"	" "	94
11-8-30	"	" "	70
11-9-30	"	" "	147
11-10-30	"	" "	38
11-11-30	"	" "	35
11-12-30	"	" "	60
11-13430	"	" "	46
11-14-30	"	" "	60
11-15-30	"	" "	80

TABLE II

Counts made on E. histolytica cultures transferred repeatedly at 48 hours.

DATE	STRAIN	AGE	NUMBER PER CU. MM.
11-5-30	W	48 hrs.	52
11-7-30	"	" "	67
11-9-30	"	" "	142
11-11-30	"	" "	50
11-13-30	"	" "	40
11-15-30	"	" "	102
11-16-30	"	" "	72
11-5-30	S	48 hrs.	48
11-7-30	"	" "	76
11-9-30	"	" "	70
11-11-30	"	" "	34
11-13-30	"	" "	74
11-15-30	"	" "	60
11-17-30	"	" "	38

TABLE III

Counts made from E. histolytica cultures repeatedly transferred at 72 hours.

DATE	STRAIN	AGE	NUMBER PER CU.MM.
11-6-30	W	72 hrs.	53
11-9-30	"	" "	5
11-12-30	"	" "	12
11-15-30	"	" "	23
11-18-30	"	" "	10
11-21-30	"	" "	16
11-24-30	"	" "	4
11-6-30	S	72 hrs.	34
11-9-30	"	" "	20
11-12-30	"	" "	37
11-15-30	"	" "	10
11-18-30	"	" "	12
11-21-30	"	" "	6
11-24-30	"	" "	8

TABLE IV

Counts on the same E. histolytica cultures taken on successive days.

DATE	STRAIN	AGE	NUMBER PER CU.MM.
11-4-30	S	24 hrs.	27
11-5-30	"	48" "	48
11-6-30	"	72 "	37
11- 7-30	"	96 "	4
11-8-30	"	120 "	0
11-4-30	"	24 "	22
11-5-30	"	48 "	52
11-6-30	"	72 "	53
11-7-30	"	96 "	6
11-5-30	S	24""	27
11-6-30	"	48 "	34
11-7-30	"	72 "	26
11-8-30	"	96 "	5
11-12-30	"	24 "	35
11-13-30	"	48"	82
11-14-30	"	72 "	67
11-15-30	"	96 "	8

II. THE STUDY OF BACTERIA ISOLATED FROM CULTURES OF ENDAMOEBA HISTOLYTICA.

PURPOSES:

1. To determine the kinds of bacteria with which the amoeba grow.
2. To study cultural reactions and find the nature of the activities of the bacteria on which E. histolytica depends.
3. To separate some strains in the attempt to find one or two strains with which the amoeba would grow with the exclusion of the others.
4. To study the pigment production of strains of Pseudomonas isolated from E. histolytica cultures.
5. To test the toxicity and pigment production of these strains of Pseudomonas when grown with other organisms.

MATERIALS USED:

Media: Plain beef extract agar, plain beef extract broth, peptone broth, gelatin, nitrate broth, litmus milk, dextrose broth, lactose broth, eosin-methylene-blue plates, and potato starch media.

All these media were made by the common laboratory formulae. The more rarely used starch medium was made by the following method: 150 gms. of peeled potatoes are boiled in one liter of water until the potatoes are done but not mushy. Filter and make up to one liter. 1% dextrose is added and 1% agar. When this is dissolved, tube in 5 to 7 cc. lots and autoclave at 15 pounds for 15 minutes. Cool and slant.

Erhlich's test for indol was used and checked by the sulphuric acid sodium nitrite test.

EXPERIMENTAL:

One loopful of a twenty-four hour culture of the amoeba and bacteria grown in Cleveland's medium was streaked on three plain agar plates by the successive streaking method. Various colonies were picked after forty-eight hours incubation at 37 degrees C. This was done repeatedly from both strains of amoeba, and in all, seventeen pure cultures were chosen for cultural study. Various media were inoculated, and the characteristics recorded(Table V). Identifications were made from characteristic reactions in these media. It is realized that there may be other

bacterial forms in the amoeba cultures, anaerobes, and varieties which require amoeba media for growth. No attempt was made to isolate these, since it was thought that those strains chosen would be representative in the study of the cultural characteristics of those organisms on which the life of the amoeba apparently depend.

As observed in the table (Table V), no Gram positive forms were found. These may have been destroyed by B. pyocyaneous which was present in large numbers. The liver medium was fequently colored a bright green from pyocyanin after forty eight hours growth at 37 degrees C. An interesting thing may be noted here, the fact that proteolytic organisms predominate, suggesting the fact that the amoeba probably require some of the proteolytic products resulting from the digestion of the medium for food. This suggests a digest medium, tryptic or bacterial digest medium for growth without bacteria, if some means of destroying the bacteria without injuring the cysts of E. histolytica could be found. Attempts to do this were made and the results are given in Section III.

Eleven of the isolated strains proved to be green pigment producers. A brief study was made of

the difference in pigment production in different media. Special attention was paid to the unusual behavior of some of the strains in potato-agar media. Table VI shows the variations in pigment in various media. In the potato-agar media, strains 1,2,11,14 and 16 eventually developed a green pigment which colored the media after 96 hours of incubation. No green pigment was observed in strain 17, a faint pink the only coloration exhibited, and this appeared usually at forty-eight hours. Strains 3,4,5,6, and 8 always showed a final red coloration beginning after 72 hours of incubation, although at 24 hours, these strains all showed a red slant and a green butt. Several inoculations of the potato media were made with each strain, and the color changes observed seemed to run true to strain. The rapidity of some of the color changes was also noted. In two or three hours time, a colorless tube would turn pink, or one which showed a red slant and a green butt would change to an entirely red tube.

It was thought that the red color in the potato medium may be due to acid pyocyanin. When acid is added to blue pyocyanin, acid pyocyanin which is red, is produced. This color change was first observed by Jordan (9) in the test tube, on

the addition of HCl to a tube containing pyocyanin. The variation from red to green in the potato medium by the different strains isolated from the E. histolytica cultures may be linked with metabolism and resulting differences in the pH of the medium. Changes in the medium from bacterial activity of different strains possibly account for the variations in color.

An interesting phenomenon observed here and also recorded by Meader, Robinson, and Leonard (8), may have some bearing on this problem. If pyocyanin is extracted by chloroform in an alkaline solution, corked and allowed to stand twenty-four hours, the blue pyocyanin in the chloroform turns green. If an alkali is added, a red, water-soluble, chloroform-insoluble dye is seen which on the addition of an acid is re-converted into green. If the chloroform extraction is not allowed to stand the twenty-four hours, it behaves as usual, turning blue with alkali and red with acid. Whatever factor is responsible for this remarkable difference in reaction, may also be the factor which causes the change in the potato medium.

It may also be brought out that the organisms which do not produce color in plain agar and exhibit very little pyocyanin are of the

smooth type of colony formation as observed on plain agar plates; and those which have considerable pyocyanin are of the rough type. It was also interesting to note that the cultural characteristics as recorded in Table V on the Pseudomonas group and their colony types, whether rough or smooth, corresponded rather closely with pigment production.

In 1925, Meader, Robinson and Leonard (8) announced the presence of a pigment which they called pyorubrin, a red, water-soluble, chloroform-insoluble pigment usually present in all strains of B. pyocyaneus. This red pigment was unchanged by the addition of acid or alkali. According to these workers, it may be demonstrated by growing B. pyocyaneus in beef-extract broth, extracting the pyocyanin with chloroform in an alkaline solution, and a red pigment in the insoluble portion indicated pyorubrin. Experiments were carried out according to the directions of these workers, but pyorubrin could not be demonstrated.

It had been noted from time to time in this laboratory, that the pyocyanin of various strains of B. pyocyaneus, seemed to be increased when in contact with other bacteria for a time. Whether the strains of Pseudomonas isolated from

E. histolytica cultures would produce more pyocyanin in the presence of certain laboratory strains of various bacteria, and whether the Pseudomonas would be toxic for these strains motivated the next series of experiments.

Plain agar plates were made with one streak for the inoculation of the organism, and a streak of various strains of Pseudomonas was made at right angles to the organism first inoculated. The toxic and pigment producing effect could be noted at the juncture of the tow lines. All of the Pseudomonas strains had been grown on plain beef extract agar for two months, and had lost much of their ability to produce pyocyanin in this medium.

The rough type of Pseudomonas, as represented by Strain 8 was the only one which showed marked inhibitory effect on any of the organism streaked with it, or which showed an increase in pigment production. With Strain 8 as shown in Table VII, inhibition of S. aureus, B. coli, strains 7 and 12, and of the mixed culture was observed. Pigment seemed to be produced with Strain 8 and the above named organisms at the point of contact. No pigment

was formed by the contact of Strain I with any of the organisms, although inhibition of B. coli, S. aureus, Strain 12, and the mixed streak was noted when in contact with Strain I.

SUMMARY:

1. Members of the B. coli, Cloacae, Flavobacterium, and Pseudomonas groups were isolated.
2. It was found that the prodominate type of bacteria growing with the amoeba was of the Pseudomonas group, probably because of the toxic effect of these strains on other organisms.
3. The organisms were highly proteolytic, pointing to the probability that E. histolytica requires protein split products in its life activities.
4. The isolated strains of Pseudomonas produced some interesting color changes in potato-agar media.
5. The production of pyocyanin seemed to correspond with the rough type of colony formation, the smooth colonies showing little pigment.
6. Pigment production and toxic effect of the Pseudomonas strains on a few laboratory pure cultures varied considerably.

EXPLANATION OF THE FOLLOWING TABLES:

TABLE V : Shows the cultural characteristics of those organisms which were isolated from cultures of E. histolytica grown in Cleveland's medium.

TABLE VI : Shows pigment production of the isolated strains of Pseudomonas in various media.

TABLE VII: Shows toxic effect and pigment production of the isolated strains of Pseudomonas when in contact with other bacteria.

TABLE V.

The cultural characteristics of bacteria isolated from E. histolytica cultures.

CULTURE NUMBER	MORPHOLOGY	GRAM	MOTILITY	DEX.	LACL	LIT. MILK	GEL.	INDOL	NITRATE	NAME
10	rod	-	≠	AG*	AG	≠ @coag	-			B.coli
13	"	"	"	"	"	" "	"			"
15	"	"	"	"	"	" "	"			"
7	"	"	"	"	"	" "	≠			B.cloacae
12	"	"	≠	-	-	- -	-			Flavobacteriaceae
2	rod	-	≠	-	-	coag&pep	≠	-	≠	
4	"	"	"	"	"	" "	"	≠	"	
5	"	"	"	"	"	" "	"	"	"	
6	"	"	"	"	"	" "	"	"	"	Members
8	"	"	"	"	"	" "	"	"	"	of
9	"	"	"	A	"	" "	"	"	"	
11	"	"	"	"	"	" "	"	"	-	Pseudomonas
1	"	"	"	"	"	" "	"	-	≠	
3	"	"	"	-	"	" "	"	≠	"	Group
17	"	"	"	A	"	" "	"	"	-	
14	"	"	"	"	"	" "	"	"	≠	
16	"	"	"	"	"	" "	"	"	"	

* Legend

- No reaction
≠ Positive reaction

A Acid

G Gas

TABLE VI

The pigment production of isolated strains of Pseudomonas in various media.

CULTURE NUMBER	PLAIN AGAR 24	48	72	96*	PEPTONE 24*	POTATO AGAR 24	48	72	96*	BEEF BROTH	COLONY
1	-	-	-	G#	G	-	-	-	G	GY	Smooth
2	-	-	-	G	G	-	-	-	G	GY	"
3	-	-	-	-	G	R$ G	R G	R R	R R	YG	Rough & Smooth
4	-	G	G	G	G	R G	R G	R -	R R	BG	Rough
5	G	G	G	G	G	R G	R G	R -	R R	BG	Rough
6	-	G	G	G	G	R G	R G	R R	R R	YG	Rough
8	G	G	G	G	G	R G	R G	R -	R R	BG	Rough
11	G	G	G	G	G	R -	R R	R G	G G	BG	Smooth
14	-	-	-	-	G	R -	R -	R -	G -	YG	Smooth
16	-	-	-	-	G	- -	-	-	G -	YG	Smooth
17	-	-	-	-	G	- -	R	-	-	Y	Smooth

* Hours of incubation at 37degreesC

\# Legend
- Colorless
G Green
R Red
Y Yellow
B Blue
G Pale Green

$ In the record of the reactions in potato agar, the top letter refers to the color of the slant, and the lower letter to the color of the butt of the tube

TABLE VII

Toxic effect and pigment production of strains of Pseudomonas with other bacteria.

Pseudomonas Culture Number	Organism streaked on plate	Pigment at 72 hours	Inhibition at 72 hours.
1	B. anthracis	- *	-
1	S. aureus	-	Sl.
1	S. tetragenous	-	-
1	M. catarrhalis	-	-
1	B. coli	-	Sl.
1	Strain 12	-	/
1	Strain 7	-	-
1	B. cloacae	-	-
1	B. alcaligines	-	-
1	B. vulgaris	-	-
1	Mixed *	-	Sl.
8	B. anthracic	-	-
8	S. aureus	/	Sl.
8	B. coli	-	Sl
8	B. vulgaris	-	-
8	Strain 7	/	Sl.
8	Strain 12	/	Sl.
8	B. cloacae	-	-
8	M. catarhalis	-	-
8	Mixed	/	Sl.
3	B. anthracis	-	-
3	S. aureus	-	-
3	S. tetragenous	-	-
3	M. catarrhalis	-	-
5	B. anthracis	-	-
5	S. aureus	-	-
5	S tetragenous	-	-
5	M. catarrhalis	-	-
14	B. anthracis	-	-
14	S. aureus	-	-
14	S. tetragenous	-	-
14	M catarrhalis	-	-

* Legend

- no reaction
/ positive reaction
Sl. Slight reaction
Mixed Bacteria from the E. histolytica cultures made directly from a 24 hour culture

Strains 7 and 12 were those isolated from amoeba cultures.

III. THE STUDY OF THE EFFECTS OF CERTAIN CHEMICAL AND PHYSICAL FACTORS ON THE CYSTS OF E. HISTOLYTICA.

PURPOSES:

1. To study the effects of certain chemicals; phenol, acriflavine, formalin, mercuric chloride, hydrochloric acid, sodium hydroxide; and the physical factors of sunlight and drouth on cysts of E. histolytica with these three purposes in view:
   a. To determine the extent of the resistance of the cysts of E. histolytica.
   b. To rid the amoeba if possible of bacteria without injury to the cysts.
   c. To compare germicidal properties of these agents on bacteria grown under the conditions of the experiment.

MATERIALS USED:

1. Phenol: Mallinckrodt's C.P.
2. Acriflavine: Abbott Laboratory product, in tablet form, in such a concentration that one tablet in one ounce of water

equalled 1-1000 dilution.

3. Formalin: Mallinckrodt's C.P.

4. Mercuric chloride: Mallinckrodt's C.P. A saturated solution of mercuric chloride was used from which dilutions were made.

5. Hydrochloric acid: Mallinckrodt's C.P.

6. Sodium hydroxide: Mreck's C. P.

TECHNIQUE:

Since no experimental work could be found with a technique given for the study of the effect of chemicals on the cysts of E. histolytica, other than the microscopic technique given by Heathman(16), a technique was devised whereby if the cysts were not injured, they would be expected to exhibit some signs of life by cultural growth. The technique as given was followed in the study of all the chemicals uced.

The sediment from a varying number of forty-eight hour old cultures of E. histolytica was placed in a sterile centrifuge tube and washed three times with sterile physiological saline. The amount of sediment depended on the extent of the experiment and on the number of inoculations which were to be made. Five tenths of a cubic centimeter was allowed for one inoculation. If four tubes were to be inocu-

lated from the sediment in one treated tube, at least two cubic centimeters of sediment were required. About 10 c.c. of the diluted reagent was then placed in the tube with the sediment. The time was taken; the tube was centrifuged; five tenths of a cubic centimeter was removed at a previously determined time interval, and placed immediately into sterile saline, washed three times by centrifugation, then inoculated into sterile media. Frequently it was allowed to stand a few minutes in the second washing until a second five tenths of a cubic centimeter could be removed at the next time interval. At first, five and ten minute time intervals were used, with unsatisfactory results since not enough time was allowed for manipulation. Finally it was decided that fifteen minutes was the lest time that the procedure could be carried through properly.

The media used for the inoculation of the treated cysts were these:

1. Sterile Cleveland's medium.
2. Sterile Cleveland's medium inoculated with a culture of mixed bacteria taken from a culture of E. histolytica, but which was entirely free from amoeba.

3. Plain agar plates.

The sterile Cleveland's medium was inoculated on the chance that the cysts would grow without bacteria, or would continue to grow by subculture with the few bacteria remaining after less strenuous treatments.

Cleveland's medium inoculated with a mixed culture was used because a favorable environment for the cysts, should any survive, was provided and injury to the cysts could be checked. If amoeba were found in this tube they would necessarily have survived treatment.

Plain agar plates were used to check on bacterial growth after treatment. It is realized that some bacteria might be left undetected by this medium, anaerobes and those which might require the liver medium for growth. However the plain agar was used as an indication of the relative bactericidal power of each reagent. Then too, bacteria which might grow only in liver could be found in the sterile Cleveland's medium after inoculation of the treated sediment.

After treatment with acid and alkali, neutralization with n/10 NaOH and n/10 HCl using neutral red as an indicator was effected before inoculation.

EXPERIMENTAL AND RESULTS:

PHENOL:

As shown in Table VIII, bacteria persist even after treatment with a five percent solution of phenol for fifteen minutes, and the amoeba are destroyed. The two percent solution of phenol in in the time used, does not kill the bacteria nor does it destroy the amoeba, although it seems to injure them. Subcultures were made from the cultures exposed to the lowest percencages of phenol. Those inoculated directly after exposure, into sterile media soon died out completely, never occurring longer than four transfers. The cysts inoculated directly after treatment into the previously inoculated media grew somewhat better, but very poorly compared to the untreated cysts of the control. This seems to indicate that the phenol must injure the cysts, or they would have survived in the inoculated Cleveland's medium, since this furnishes a favorable environment for growth. The fact that they died earlier in the sterile Cleveland's medium, points to the probability of lack of nutriment. Possibly the bacteria on which they depended were destroyed by the treatment.

Table VIII, which follows, gives the results of the experiment in detail.

TABLE VIII.

The effect of phenol in varying dilutions on E. histolytica and bacteria.

DILUTION OF PHENOL	15 MINUTES EXPOSURE AT 20° C.		30 MINUTES EXPOSURE AT 20° C.		MEDIA
	EXAMINATION AT 24 Hrs.	48 HRS.	EXAMINATION AT 24 HRS.	48 HRS.	
1-20	-Am *	-Am	-Am	-Am	A
5%	-Am	- Am	-Am	- Am	B
	Few Bac.	Few Bac.	-	-	C
1-50	Few Am.	Man. Am.	Few Am.	Few Am.	A
2%	Man.Am.	Man. Am.	Man.Am.	Man. Am	B.
	Few Bac.	Few Bac.	Few Bac.	Few Bac.	C.
1-500	Few Am.	Few Am.	- Am.	Few Am.	A
0.2%	Man.Am.	Man.Am.	Man. Am.	Man. Am.	B
	Man. Bac.	Man. Bac.	Man. Bac.	Man. Bac.	C
1-1000	MAN.AM.	Man. Am.	Man. Am.	Man. Am.	A
0.1%	Man.Am.	Man. Am.	Man. Am.	Man. Am.	B
	Man. Bac.	Man. Bac.	Man. Bac.	Man. Bac.	C

Controls
1. Cysts without treatment in A--- Growth
2. Cysts without treatment in B--- Growth

*Legend

- No growth
Am- Amoeba
Man.- Many
Bac.- Bacteria

Media A- Sterile Cleveland's Medium
Media B- Cleveland's medium inoculated with mixed bacteria from E. histolytica cultures
Media C-Plain Sterile agar plates.

ACRIFLAVINE:

Table IX shows the results obtained by exposing the sediment containing E. histolytica to varying dilutions of acriflavine.The acriflavine in high dilutions seemed to affect the amoeba more readily than the bacteria, since bacteria were found on all agar plates of all dilutions in all time intervals used. In addition to the fifteen and thirty minute periods, the sediment was also exposed to two hours and also five hours in a 1-500 dilution of acriflavine. Bacteria were also found on the plates streaked from these treatments. As shown in Table IX, some amebic cysts but no trophic forms in both sterile and inoculated Cleveland's media in the 1-1000, 1-5000, and 1-10,000 dilutions of acriflavine. These were placed in subculture for two weeks. Those exposed in 1-5000 and in 1-10,000 survived but grew rather poorly. As a result of these experiments, it would seem that acriflavine is not a very effective disinfectant for albuminous material. The cysts seem to withstand the high dilutions, but the lower dilutions kill them. This reagent proved unsatisfactory for the purposes in mind.

The following Table IX gives the results in detail.

TABLE IX.

The effects of Acriflavine in varying dilutions on E. histolytica and bacteria.

DILUTIONS OF ACRIFLAVINE	15 MINUTES AT 20° C.		30 MINUTES AT 20° C.		MEDIA
	EXAMINATION AT 24 HRS.	48 HRS.	EXAMINATION 24 HRS	48 HRS	
1-100	-Am.*	-Am.	-Am	-Am	A
	-Am	-Am	-Am	-Am	B
	Few Bac	Few Bac	Few Bac	Few Bac	C
1-500	-Am	-Am	Few Am	-Am	A
	-Am	-Am	Few Am	-Am	B
	Man.Bac.	Man Bac	Man Bac	Man Bac	C
1-1000	-Am	-Am	-Am	-Am	A
	Few Am	Few Am	Few Am	Few Am	B
	Man Bac	Man Bac	Man Bac	Man Bac	C
1-5000	Few Am	Few Am	Few Am	Few Am	A
	Few Am	Few Am	Few Am	Few Am	B
	Man Bac	Man Bac	Man Bac	Man Bac	C
1-10,000	Man Am	Man Am	Man Am	Man Am	A
	Man Am	Man Am	Man Am	Man Am	B
	Man Bac	Man Bac	Man Bac	Man Bac	C
Controls	1. Cysts without treatment into A----Growth				
	2. Cysts without Treatment into B.---- Growth				

* Legend

- No growth
Am- Amoeba

For media see Table VIII Legend
Bac- Bacteria
Man- Many (more than 1 per field L.P.)
Few- Few (Less than 1 per field L.P.)

MERCURIC CHLORIDE:

Table X shows the results of treatment with mercuric chloride. This did not prove as satisfactory a bactericidal agent as phenol, the bacteria surviving a 1-500 dilution of mercuric chloride for fifteen minutes. The cysts found after this treatment and incubation in media, as indicated in Table X were either dead or injured as found in subsequent failure to grow in subculture. Subcultures were made from the cultures exposed to 1-1000 mercuric chloride, but after four transfers, no more amoeba were found. This reagent seems to show more possibilities than any of the others studied, since the amoeba, as shown by the table were found after exposure to lower dilutions than the other reagents.

TABLE X.

The effect of mercuric chloride in varying dilutions on E. histolytica and bacteria.

DILUTIONS OF MERCURIC CHLORIDE	15 MINUTES 20° C. EXAMINATION AT 24 HRS	48 HRS	30 MINUTES 20° C EXAMINATION AT 24 HRS	48 HRS	MEDIA
	- Am *	- AM	- AM	- AM	A
1-100	Few Am	Few Am	- Am	- Am	B
	- Bac	- Bac	- Bac	- Bac	C
	Few Am	Few Am	Few Am	- Am	A
1-500	Few Am	Few Am	Few Am	Few Am	B
	Man Bac.	Man Bac	Man Bac	Man Bac	C
	Man Am	Man Am	Man Am	Man Am	A
1-1000	Man Am	Man Am	Man Am	Man Am	B
	Man Bac	Man Bac	Man Bac	Man Bac	C
	Man Am	Man Am	Man Am	Man Am	A
1-5000	Man Am	Man Am	Man Am	Man Am	B
	Man Bac	Man Bac	Man Bac	Man Bac	C
	Man Am	Man Am	Man Am	Man Am	A
1-10,000	Man Am	Man Am	Man Am	Man Am	B
	Man Bac	Man Bac	Man Bac	Man Bac	C

Controls

1. Media A inoculated with untreated Amoeba--Growth
2. Media B inoculated with untreated amoeba--Growth

*Legend

- No growth
Am Amoeba
Bac Bacteria
Man. Many(More than 1 per field L.P.) More than 10 colonies per plate
Few (Less than 1 per field L.P.) Less than 10 colonies per plate

Media A Sterile Cleveland's
Media B. Cleveland's inoculated with a mixed culture
Media C Plain agar plates.

FORMALIN:

Table XI shows the results on E. histolytica of treatment with varying dilutions of formalin. This did not prove to be a very effective germicide in the lower dilutions with short time exposure, but the cysts were injured to the extent of persisting no longer than three subcultures after treatment. The higher dilutions with longer exposure were tested for use in making a vaccine. The cysts were exposed to 0.1%, 0.2%, and 0.5% formalin at room temperature (20 degrees C.) and at ice box temperature (6-8 degrees C.) for twenty-four hours. The three media were used as with the other reagents. No difference could be seen in the reactions at the two temperatures. No trophic forms were seen, but cysts were observed. As shown in the table, bacteria were found in those cultures exposed to 0.1% formalin. The cysts were thought to be dead or injured since they failed to grow in further subculture. The cysts found after exposure to 0.2% formalin were also thought to be dead since none of these survived subsequent subculture. No trophic forms were seen. Therefore it was determined that twenty-four hours exposure at either 20 degrees or 6 to 8 degreesC. destroyed bacteria and cysts.

TABLE XI

The effect of formalin in varying dilutions on E. histolytica and bacteria.

DILUTION of FORMALIN	15 MINUTES EXPOSURE AT 20° C. EXAMINATION AT 24 Hours		30 MINUTES EXPOSURE AT 20° C. EXAMINATION AT 24 HRS		MEDIA
	24 Hours	48HRS	24 HRS	48 HRS	
01%	Man Am*	Man Am	Man Am	Man Am	A
	Man Am	Man Am	Man Am	Man Am	B
	Man Bac	Man Bac	Man Bac	Man Bac	C
0.5%	Man Am	Man Am	Man Am	Man Am	A
	Man Am	Man Am	Man Am	Man Am	B
	Man Bac	Man Bac	Man Bac	Man Bac	C
	24 Hours at 6-8° C EXAMINATION AT 24 HRS	48 HRS	24 HOURS at 20° C EXAMINATION AT 24 HRS	48 HRS	
0.1%	Few Am.	Few Am	Few Am	Few Am	A
	Few Am	Few Am	Few Am	Few Am	B
	Man. Bac	Man Bac	Man Bac	Man Bac	C
0.2%	Occ. cyst	Occ cyst	Occ cyst	Occ cyst	A
	Occ cyst	Occ cyst	Occ cyst	Occ cyst	B
	- Bac	- Bac	- Bac	- Bac	C
0.5%	Occ cyst	Occ cyst	Occ cyst	Occ cyst	A
	Occ cyst	Occ cyst	Occ cyst	Occ cyst	B
	- Bac	- Bac	- Bac	- Bac	C
Controls	1. Untreated amoeba into A-- Growth				
	2. Untreated amoeba into B-- Growth				

*Legend

Man- Many(More than 1 per field L.P. More than 10 colonies per plate)
Few- Few (Less than 1 per field L.P. Less than 10 colony per plate)
Occ. Occasional (1 every 4-5 fields L.P.)
- No Growth
Am- Amoeba
Bac. Bacteria

Media A. Sterile Cleveland's
Media B. Cleveland's inoculated with a mixed culture
Media C. Plain agar plates

HYDROCHLORIC ACID:

Table XII shows the results on E. histolytica of treatment with varying dilutions of hydrochloric acid. As given under technique, the sediment was carefully neutralized with n/10 NaOH after exposure to HCl, before inoculation. The cysts found after twenty-four hours incubation were dead or injured as shown by subsequent failure to grow in subculture. The ones exposed to one percent acid for fifteen minutes survived four subcultures in both media and then died out. However bacteria survived at this dilution therefore this is not a very effective reagent for the objective in view.

This treatment was used because Dobell and Laidlaw (3) suggest a 0.2% HCl exposure of two hours seems to benefit the amoeba by destroying some of the forms of bacteria which grow with them.

TABLE XII.

The effect of hydrochloric acid in varying dilutions on E. histolytica and bacteria.

DILUTION OF HCL	15 MINUTES AT 20° C. EXAMINATION AT 24 HRS	48 HRS	30 MINUTES AT 20° C. EXAMINATION AT 24 HRS	48 HRS	MEDIA
01.0%	*Few AM.	-Am	Few Am	-Am	A
	Few Am	Few Am	Few Am	Few Am	B
	Few Bac	Few Bac	Few Bac	Few Bac	C
05.0%	Few Am	-Am	-Am	-Am	A
	Few Am	-Am	-Am	-Am	B
	Few Bac	Few Bac	-Bac	-Bac	C
10.0%	Few Am	-Am	-Am	-Am	A
	Few Am	-Am	-Am	-Am	B
	-Bac	-Bac	-Bac	-Bac	C

* Legend

- No Growth
Am Amoeba
Bac -Bacteria
Few- Less than one per field L.P.
Less than 10 colonies on plate.
Media A Sterile Cleveland's
Media B Sterile Cleveland's inoculated with mixed bacteria from culture of E. histolytica
Media C Plain agar plate

SODIUM HYDROXIDE

Table XIII shows the effect of treatment with sodium hydroxide in varying dilutions on E. histolytica and accompanying bacteria. After exposure and incubation in the media used no amoeba, cysts or trophic forms were found in any dilution used. Bacteria were found after fifteen minutes exposure to one percent NaOH. The untreated cysts inoculated into media showed normal growth. These last experiments seem to show that cysts seem to withstand acid better than alkali, and that the lower dilutions of NaOH are an effective germicide.

TABLE XIII

The effect of sodium hydroxide in varying dilutions on E. histolytica and bacteria.

DILUTIONS OF NAOH	15 MINUTES AT 20° C. EXAMINATION AT 24 HRS	48 HRS	30 MINUTES AT 20° C. EXAMINATION AT 24 HRS	48 HRS	Media
1.0 %	-Am *	-Am	-Am	-Am	A
	-Am	-Am	-Am	-Am	B
	Few Bac.	Few Bac	Few Bac	Few Bac	C
5.0%	-Am	-Am	-Am	-Am	A
	-Am	-Am	-Am	-Am	B
	-Bac	-Bac	-Bac	-Bac	C
10.0%	-Am	-Am	-Am	-Am	A
	-Am	-Am	-Am	-Am	B
	-Bac	-Bac	-Bac	-Bac	C
25.0%	-Am	-Am	-Am	-Am	A
	-Am	-Am	-Am	-Am	B
	-Bac	-Bac	-Bac	-Bac	C

* - No growth

Am Amoeba

Bac Bacteria

Few-- Less than 1 per field L.P.
Less than 10 colonies per plate

Media A. Sterile Cleveland's
Media B. Cleveland's inoculated with mixed culture of bacteria from E. histolytica culture.
Media C. Plain agar plates.

SUNLIGHT:

One cubic centimeter of sediment of a forty-eight hournculture of E. histolytica, suspended in saline, was exposed to sunlight in sterile petri dishes, the lids remaining on the dishes for the interval of exposure. Two intervals of time were used, four and six hours. Both bacteria andamoeba survived the treatment, the four hour exposure as well as the six hour one. No effect at all was seen on the culture as compared with the control. The cultures were carried for two weeks and dropped. Further work with this and ultra violet ray might prove interesting.

DROUGHT

The cysts of E. histolytica which were dried for twenty-four hours were inoculated into sterile Cleveland's medium and into a tube of the liver-agar which had been previously inoculated with a mixed culture of bacteria from a culture of E. histolytica. No amoeba were seen, either cysts or trophic forms. The drying seemed to destroy them completely.

VITAL DYES:

As reported in the literature section, Browne and Donavan (13) found neutral red and Janus green dyes were non toxic to E. histolytica up to a one percent solution. However in this work, it was found that neither Strain S nor W resisted either neutral red or Janus green made up in absolute alcohol to a concentration of one percent. With a four tenths alcoholic solution of neutral red, the vacuoles stained pink, the starch granules remained unstained enclosed in a pink vacuole. The nucleus stained pink surrounded with an unstained cytoplasm. These sealed preparations were made according to the technique of Sabin given in Todd(14) for supra-vital staining in blood work. The slides were thoroughly cleansed with acid, rinsed in tap water, then distilled water, stored in absolute alcohol, and finally flamed with alcohol before flooding with the dye. A drop of the amoeba culture was placed on the slide, a clean cover slip placed on the drop, and sealed with a paraffin-vaseline mixture. A warm stage was first used, but later abandoned when it was found that the amoeba remained motile for twenty four hours, either in the warm stage or at room temperature. This motility was exhibited both in the presence of the dye and on the clean glass slide sealed as in the dye preparation.

SUMMARY:

1. Phenol proved to be an effective germicide after thirty minutes exposure to a five percent solution. Th The amebic cysts seemed to survive a two percent solution for thirty minutes exposure, but may have been injured as shown by failure to continue to grow in subculture.

2. Acriflavine was not an effective germicide in any dilution used. The cysts resisted a 1-5000 dilution but did not grow in subculture.

3. Mercuric chlorode in a 1-100 dilution after fifteen minutes exposure destroyed the bacteria, but the amoeba were also destroyed. The amebic cysts survived the 1-1009 dilution after an exposure of thirty minutes, but did not survive subsequent subcultures.

4. Formalin was not effective as a germicide in the low dilutions used for short time exposure. Twenty-four hours exposure in a 0.2% dilution is effective.

5. A ten percent solution of HCl with a fifteen minute exposure is an effective germicide, but a five percent solution for fifteen minutes will destroy amoeba.

6. Sodium hydroxide in a dolution of one percent destroys cysts, but bacteria require a five percent solution to be destroyed.

7. The cysts of E. histolytica resist sunlight exposure six hours with the technique described, as do most of the bacteria present.

8. Drying twenty-four hours destroys E. histolytica.

9. A film prepared on the slide from a one percent alcoholic solution of Janus green and likewise a film form a one percent solution of neutral red caused the destruction of E. Histolytica within a few seconds.

10. A film prepared on the slide from a four tenths alcoholic solution of Janus green or of neutral red seemed to have little toxicity, the organisms remaining motile for twenty-four hours.

11. A film prepared on the slide from a four tenths solution of Janus green or of neutral red in absolute alcohol, is effective as a stain for the living organism. (see page 47 for technique).

12. Trophic forms of E. histolytica remained motile in a sealed preparation, from twenty-four to forty-eight hours at 20 degrees C., and at 37 degrees C, either in the presence of the vital dyes or in the unstained preparations.

IV. A BRIEF STUDY OF SOME COMPLEMENT-FIXATION TESTS WITH E. HISTOLYTICA AND BLASTOCYSTIS HOMINIS.

PURPOSES:

1. To study complement-fixation with E. histolytica and Blastocystis hominis using: E. histolytica immune serum, and homologous antigen; B hominis immune serum and homologoud antigen; E. histolytica immune serum and B. hominis antigen; B. hominis immune serum and E. histolytica antigen.
2. To compare the reactions af alcoholic lipoid antigens prepared from E. histolytica and B. hominis, and saline extract antigens prepared from these organisms.
3. To test normal sera for positive reations with the alcoholic lipoid antigens.

MATERIALS USED:

1. Organisms:

   a. E. histolytica: The strains used in this part of the work were the same as those described in the first section.

in all cases, the strains S and W were mixed in the preparation of antigens.

b. Blastocystis hominis: This intestinal vegetative form of the plant kingdom was cultured from the stool of a student who showed a four plus complement-fixation test with the antigen prepared by Sherwood (see Antigen below). Later the organism was found in the stools of several students who gave a four plus fixation with no evidence of amebiasis. The method of isolation and culture of B. hominis was the same as for E. histolytica. A loopful of fecal material was inoculated directly into a tube of sterile Cleveland's medium. In forty-eight hours, a heavy growth of Blastocystis could be seen. After seven days without transfery there were still many organisms in the culture, and they would grow readily in subculture.

B. hominis was found to grow not only in the liver-agar-horse-serum media, but equally as well in the Boeck-Drobohlav coagulated egg medium. Contrary to Dobell and Laidlaw's report(3) that rice flour prevents growth, B. hominis appeared not to be affected at all either by the presence or absence of rice flour. An attempt was made to grow B.

hominis in all the common laboratory media. It failed to grow in the various sugar media, maintaining itself fairly well for a short time in peptone and in plain broth. Wenyon (15) reports that these organisms may be cultured from any normal stool, using the media prepared for E. histolytica. B. hominis is very much like the amebic cyst in form, and it was thought that this organism, as a contaminate in the cultures of E. histolytica might play a part in the fixation of complement by apparently normal sera.

2. Antigens:

The method of preparation of the alcoholic lipoid antigen was taken from Heathman(16). No allowance was made for the presence of bacteria. Sherwood uses an antigen which is more concentrated. His procedure as given in an unpublished work is to use from about one-half to one third the amounts of alcohol and acetone as used by Heathman, and to include a period of shaking to facilitate the extraction.

The method of making antigen by saline extraction was also taken from Heathman(16). The washed, packed amoebae were dried. The washing consisted of three centrifugations

with sterile normal saline. The dried amoebae were then triturated with dry NaCl in a sterile agate mortar in the proportion of one tenth gram of dried cells to 0.17 gram of salt. After extracting in this way for one hour, distilled water was gradually added, and the trituration continued until the suspension was finally made up to isotonicity in another hour. Antigens for both E. histolytica and B. hominis were made by this method. In the case of the latter organism, it was necessary to run the centrifuge at full speed, to throw down the organism.

3. Animals:

All animals used, both guinea pigs and rabbits were apparently healthy, normal, and adult.

TECHNIQUE:

1. Animal inoculation:

a. E. histolytica: In finding a non toxic dose of E. histolytica for immunization, a number of rabbits succumbed. Finally, the following procedure was adopted: The sediment from two forty-eight hour cultures was washed three times in sterile, normal saline; five tenths cubic centimeter of these washed packed cells were added

to ten cubic centimeters of physiological saline containing four tenths percent phenol. This was allowed to stand in the ice-box twenty-four hours before using; five tenths cubic centimeters of this suspension made up to five cubic centimeters in normal saline and inoculated intraperitoneally proved non toxic. This initial dose was calculated to contain about 1000 amebic cysts. Five inoculations were given at three day intervals, in amounts of 1.0, 1.5, 2.0, 2.5, cc, each made up to five cc with sterile physiological saline. The animal was bled after a ten day interval, and the serum tested as given under procedure.

b. Blastocystis hominis:

Again in finding a non toxic dose, several animals succumbed. The saline extract antigen, as used by Heathman(16) was tried and proved non toxic. Five doses were given at three day intervals in five cubic centimeter quantities, intraperitoneally. Ten days were allowed to elapse and the animal was bled. The serum was tested as given under procedure below.

PROCEDURE:

TEST I.

To test the antigen six dilutions were made, 1-5, 1-10, 1-20, 1-30, 1-50, 1-100. Five tneths of a cubic centimeter each of a one to five dilution of four sera were set up perar-ately with the above dilutions of antigen. The four sera used were; a serum from a rabbit inoculated with E. histolytica, a serum from a rabbit inoculated with B. hominis, a normal human serum showing a four plus fixation with Sherwood's alcoholic lipoid antigen III, and a normal human serum showing no fixation with Sherwood's antigen.

The tables which follow, XIV to XIX, show the results of this experiment. Fixation was obtained in those tubes which contained the positive immune serum produced against the amoeba. It was apparently very high titred. No further effort was made to obtain the exact titre. The failure to obtain fixation with positive normal serum may have been due to the low concentration of the antigen. As described under preparation of antigen, Sherwood's antigenIII is highly concentrated.

TABLE XIV.

E. histolytica immune serum and varying amounts of E. histolytica alcoholic lipoid antigen.

E.HIST. LIPOID ANTIGEN DILUTION	AMOUNT	RABBIT ANTI AMOEBA SERUM	COMPLEMENT 2 FULL UNITS	NACL		SHEEP CELLS 2%	HEMOLYSIN 2 FULL UNITS	RESULTS
undil	.5	.5	.5	.5		.5	.5	++++ *
1-5	.5	.5	.5	.5		.5	.5	++++
1-10	.5	.5	.5	.5	incubation	.5	.5	++++
1-20	.5	.5	.5	.5	in ice	.5	.5	++
1-30	.5	.5	.5	.5	box 18 hours	.5	.5	++
1-50	.5	.5	.5	.5	After sensitized	.5	.5	+
1-100	.5	.5	.5	.5	cells added one	.5	.5	+
Controls 1-10	.5	0	0	1.5	hour 37 degrees	.5	.5	-
	0	.5	.5	1		.5	.5	-
	0	.5	0	1.0		.5	.5	-
	0	0	.5	1.5		.5	.5	-

GGGG

* ++++ four plus fixation
++ two plus fixation
+ one plus fixation
- no fixation

TABLE XV

B. hominis immune serum and varying amounts of B. hominis alcoholic lipoid antigen.

B.HOMINIS LIPOID ANTIGEN DILUTION	AMOUNT	RABBIT ANTI B.HOM. SERUM	COMPLEMENT 2 FULL UNITS	NACL		SHEEP CELLS 2%	HEMOLYSIN 2 FULL UNITS	RESULTS
undil.	.5	.5	.5	.5		.5	.5	- *
1-5	.5	.5	.5	.5		.5	.5	-
1-10	.5	.5	.5	.5	Incubation	.5	.5	-
1-20	.5	.5	.5	.5	in ice box 18 hours	.5	.5	-
1-30	.5	.5	.5	.5		.5	.5	-
1-50	.5	.5	.5	.5	After cells added,	.5	.5	-
1-100	.5	.5	.5	.5	1 hour 37°C	.5	.5	-
Controls								
1-10	.5	0	0	1.5		.5	.5	-
	0	.5	.5	1.0		.5	.5	-
	0	.5	0	1.5		.5	.5	-
	0	0	.5	1.5		.5	.5	-

* - No fixation

TABLE XVI

Negative normal serum and varying amounts of B. hominis alcoholic lipoid antigen.

B.HOMINIS LIPOID ANTIGEN DIL-UTION	AM'T	NEGATIVE NORMAL SERUM	COMPLEMENT 2 FULL UNITS	NACL		SHEEP CELLS 2%	HEMOLYSIN 2 FULL UNITS	RESULTS
undil	.5	.5	.5	.5		.5	.5	- *
1-5	.5	.5	.5	.5		.5	.5	-
1-10	.5	.5	.5	.5	Incubation in ice-box	.5	.5	-
1-20	.5	.5	.5	.5	18 Hrs.	.5	.5	-
1-30	.5	.5	.5	.5	After cells	.5	.5	-
1-50	.5	.5	.5	.5	added 1 hour	.5	.5	-
1-100	.5	.5	.5	.5	37° C.	.5	.5	-
Controls								
1-10	.5	0	0	1.5		.5	.5	-
	0	.5	.5	1.0		.5	.5	-
	0	.5	0	1.5		.5	.5	-
	0	0	.5	1.5		.5	.5	-

* - No fixation

Negative normal serum refers to a normal which gave no complement fixation with the quantative technique of Sherwood, using Sherwood's antigen III.

TABLE XVII.

Negative normal serum and varying amounts of E. histolytica alcoholic lipoid antigen.

E. HIST. LIPOID ANTIGEN DIL-UTION	AM'T	NEG-ATIVE NORMAL SERUM	COMP-LEMENT 2 FULL UNITS	NACL		SHEEP CELLS 2%	HEMO-LYSIN 2 FULL UNITS	RESULTS
undil.	.5	.5	.5	.5		.5	.5	- *
1-5	.5	.5	.5	.5		.5	.5	-
1-10	.5	.5	.5	.5	Incuba-tion	.5	.5	-
1-20	.5	.5	.5	.5	in ice box	.5	.5	-
1-30	.5	.5	.5	.5	18 hrs.	.5	.5	-
1-50	.5	.5	.5	.5	After adding cells	.5	.5	-
1-100	.5	.5	.5	.5	1 hr. 370 C	.5	.5	-
Controls								
1-10	.5	0	0	1.5		.5	.5	-
	0	.5	.5	1.0		.5	.5	-
	0	.5	.5	1.5		.5	.5	-
	0	0	.5	1.5		.5	.5	-

* - No fixation

Negative normal serum refers to a normal which gave no fixation with the quantative technique of Sherwood using Sherwood's alcoholic lipoid antigen III.

TABLE XVIII.

Positive normal serum and varying amounts of B. hominis alcoholic lipoid antigen.

B.HOMINIS LIPOID ANTIGEN DILUTION	AM'T	*POSITIVE NORMAL SERUM	COMPLEMENT 2 FULL UNITS	NACL		SHEEP CELLS 2%	HEMOLYSIN 2 FULL UNITS	RESULTS
undil.	.5	.5	.5	.5		.5	.5	-*
1-5	.5	.5	.5	.5		.5	.5	-
1-10	.5	.5	.5	.5	Incubation	.5	.5	-
1-20	.5	.5	.5	.5	in ice box	.5	.5	-
1-30	.5	.5	.5	.5	18 Hrs.	.5	.5	-
1-50	.5	.5	.5	.5	After cells added	.5	.5	-
1-100	.5	.5	.5	.5	1 hr. at 37°C.	.5	.5	-
Controls								
1-10	.5	0	0	1.5		.5	.5	-
	0	.5	.5	1.0		.5	.5	-
	0	.5	0	1.5		.5	.5	-
	0	0	.5	1.5		.5	.5	-

* Positive normal serum refers to a normal with no evidence of amebiasis, which gave a four plus complement fixation test with the quantative technique of Sherwood, using Sherwood's alcoholic lipoid antigen.

\- No reaction.

TABLE XIX.

Positive normal serum and varying amounts of E. histolytica alcoholic lipoid antigen.

E. HIST. LIPOID ANTIGEN		*POSITIVE NORMAL SERUM	COMPLEMENT 2 FULL UNITS	NACL	SHEEP CELLS 2%	HEMOLYSIN 2 FULL UNITS	RESULTS
DILUTION	AM'T						
undil.	.5	.5	.5	.5	.5	.5	- *
1-5	.5	.5	.5	.5	.5	.5	-
1-10	.5	.5	.5	.5	.5	.5	-
1-20	.5	.5	.5	.5	.5	.5	-
1-30	.5	.5	.5	.5	.5	.5	-
1-50	.5	.5	.5	.5	.5	.5	-
1-100	.5	.5	.5	.5	.5	.5	-
Controls							
1-10	.5	0	0	1.5	.5	.5	-
	0	.5	.5	1.0	.5	.5	-
	0	.5	0	1.5	.5	.5	-
	0	0	.5	1.5	.5	.5	-

Incubation in ice box 18 hrs. After cells added 1 hr. at 37°C.

* Positive normal serum refers to a normal person who with no evidence of amebiasis, gave a four plus complement-fixation test, with the quantative technique of Dr. Sherwood, using Sherwood's alcoholic lipoid antigen III.

\- No fixation.

TEST 2.

Tests were set up using the quantative technique of Sherwood. First a series of tubes containing the alcoholic lipoid antigen obtained from E. histolytica, and another series containing the saline extract antigen obtained from the same source were set up with homologous antiserum. Then a series of tubes containing measured amounts of alcoholic lipoid antigen obtained from B. hominis, and another series containing the antigen obtained from B. hominis by saline extraction were set up with homologous immune serum. A one to ten dilution of antigen was used in all cases. Then the antigens from both sources were set up with the immune serum obtained from the opposite organism. The results of these experiments are given in the tables which follow, Tables XX to XXXV. As can be seen, the whole series, with the exception of the E. histolytica immune serum set up with the alcoholic lipoid antigen made from the amoeba, showed no fixation.

TABLE XX.

E. Histolytica immune serum and E. histolytica alcoholic lipoid antigen.

AMOEBA IMMUNE SERUM	NACL	E.HIST. LIPOID ANTIGEN	COMPLEMENT 2 FULL UNITS		SHEEP CELLS 2%	HEMOLYSIN 2 F.U.*	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	- *
.1	1.3	.1	.5	18 hrs.	.5	.5	++++
.5(1-10)	.9	.1	.5	Add cells	.5	.5	++++
.25(1-10)	1.15	.1	.5	1 hr 37° C.	.5	.5	+++

TABLE XXI.

E.histolytica Immune serum and E. histolytica antigen obtained by saline extraction.

AMOEBA IMMUNE SERUM	NACL	E.HIST SALINE EXTRACT ANTIGEN	COMPLEMENT 2 F.U.*		SHEEP CELLS 2%	HEMOLYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5(1-10)	.9	.1	.5	Add cells	.5	.5	-
.25(1-10)	1.15	.1	.5	1 hr. 37°C.	.5	.5	-

* F.U. -- Full Unit

\- No fixation

TABLE XXII

Blastocystis hominis immune serum and E. histolytica lipoid antigen.

B.HOMINIS IMMUNE SERUM	NACL	E.HIST. LIPOID ANTIGEN	COMP-LEMENT 2 F.U.*		SHEEP CELLS 2%	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5(1-10)	.9	.1	.5	Add Cells	.5	.5	-
.25(1-10)	1.15	.1	.5	1 hr. 37° C.	.5	.5	-

TABLE XXIII.

B. hominis immune serum and E. histolytica antigen obtained be saline extract.

B. HOMINIS IMMUNE SERUM	NACL	E.HIST ANTIGEN SALINE EXTRACT.	COMP-LEMENT 2 F.U.*		SHEEP CELLS 2%	HEMO-LYSIN 2.F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5(1-10)	.9	.1	.5	Add cells	.5	.5	-
.25(1-10)	1.15	.1	.5	1 hr 37° C.	.5	.5	-

* F.U. Full unit

\- No fixation

TABLE XXIV.

B. hominis immune serum and B. hominis alcoholic lipoid antigen.

B.HOMINIS IMMUNE SERUM	NACL	B.HOM. LIPOID ANTIGEN	COMP-LEMENT 2 F.U.*		SHEEP CELLS 2 %	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box , incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5(1-10)	.9	.1	.5	Add cells	.5	.5	-
.25(1-10)	1.15	.1	.5	1 hr. 37° C.	.5	.5	-

TABLE XXV.

B. hominis immune serum and B. hominis antigen obtained by saline extraction.

B.HOMINIS IMMUNE SERUM	NACL	B.HOM. SALINE EXT. ANTIGEN	COMP-LEMENT 2 F.U.		SHEEP CELLS 2 %	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box 18 hrs.	.5	.5	-
.1	1.3	.1	.5		.5	.5	-
.5(1-10)	.9	.1	.5	Add cells 1 hr, 37° C.	.5	.5	-
.25(1-10)	1.15	.1	.5		.5	.5	-

* F.U. - FULL UNIT

\- NO FIXATION

TABLE XXVI.

E. histolytica immune serum and B. hominis alcoholic lipoid antigen.

E. HIST. IMMUNE SERUM	NACL	B. HOMINIS LIPOID ANTIGEN	COMP-LEMENT 2 F.U.*		SHEEP CELLS 2 %	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs	.5	.5	-
.5(1-10)	.9	.1	.5	Add cells 1 hr.	.5	.5	-
.25(1-10)	1.15	.1	.5	37° C.	.5	.5	-

TABLE XXVII.

E. histolytica immune serum and B. hominis antigen obtained by saline extraction.

E. HIST. IMMUNE SERUM	NACL	B.HOM. SALINE EXT. ANTIGEN	COMP-LEMENT 2 F.U.		SHEEP CELLS 2 %	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5(1-10)	.9	.1	.5	Add cells 1 hr.	.5	.5	-
.25(1-10)	1.15	.1	.5	37° C.	.5	.5	-

* F. U. - Full unit

\- No fixation

TABLE XXVIII

Negative normal serum and E. histolytica alcoholic lipoid antigen.*

NEGATIVE SERUM	NACL	E.HIST. LIPOID ANTIGEN	COMPLEMENT 2 F.U.		SHEEP CELLS 2 %	HEMOLYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs. Add cells	.5	.5	-
.5(1-10)	.9	.1	.5	1 hr. 37° C.	.5	.5	-
.25(1-10)	1.15	.1	.5		.5	.5	-

TABLE XXIX

Negative normal serum and B. hominis alcoholic lipoid antigen.

NEGATIVE SERUM	NACL	B.HOM. LIPOID ANTIGEN	COMPLEMENT 2 F.U.		SHEEP CELLS 2 %	HEMOLYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5(1-10)	.9	.1	.5	Add cells 1 hr.	.5	.5	-
.25(1-10)	1.15	.1	.5	37° C.	.5	.5	-

* Negative normal serum refers to a normal which does not give a four plus fixation with Sherwood's antigen III.

F.U.- Full unit
\- No fixation.

TABLE XXX

Negative normal serum and B. hominis antigen obtained by saline extraction.*

NEGATIVE SERUM	NACL	B.HOM. SALINE EXT. ANTIGEN	COMP-LEMENT 2 F.U. *		SHEEP CELLS 2%	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation 18 hrs. Add cells 1 hr. 37° C.	.5	.5	-
.1	1.3	.1	.5		.5	.5	-
.5(1-10)	.9	.1	.5		.5	.5	-
.25(1-10)	1.15	.1	.5		.5	.5	-

TABLE XXXI

Negative normal serum and E. histolytica antigen obtained by saline extraction.

NEGATIVE SERUM	NACL	E.HIST. SALINE EXTRACT. ANTIGEN	COMP-LEMENT 2 F.U.		SHEEP CELLS 2%	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box 18 hrs. add cells 1 hr. 37° C.	.5	.5	-
.1	1.3	.1	.5		.5	.5	-
.5(1-10)	.9	.1	.5		.5	.5	-
.25(1-10)	1.15	.1	.5		.5	.5	-

* Negative normal serum refers to a serum which does not give a positive fixation test with Sherwood's lipoid antigen III.

F.U.- Full unit
\- No fixation

TABLE XXXII.

Positive normal serum and E. histolytica alcoholic lipoid antigen.*

POSITIVE SERUM	NACL	E.HIST. LIPOID ANTIGEN	COMP-LEMENT 2 F.U.		SHEEP CELLS 2%	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5 (1-10)	.9	.1	.5	Add cells 1 hr. 37° C.	.5	.5	-
.25(1-10)	1.15	.1	.5		.5	.5	-

TABLE XXXIII.

Positive normal serum and B. hominis lipoid antigen *

POSITIVE SERUM	NACL	E.HOM. LIPOID ANTIGEN	COMP-LEMENT 2 F.U.		SHEEP CELLS 2%	HEMO-LYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5 (1-10)	.9	.1	.5	Add cells 1 hr.	.5	.5	-
.25(1-10)	1.15	.1	.5	37° C.	.5	.5	-

* Positive normal serum refers to a normal which gives a four plus complement fixation with Sherwood's alcoholic lipoid antigen III.
F.U. full unit
- no fixation.

TABLE XXXIV.

Positive normal serum and B. hominis antigen obtained by saline extraction. *

POSITIVE SERUM	NACL	B.HOM. SALINE EXT. ANTIGEN	COMPLEMENT 2 F.U.*		SHEEP CELLS 2 %	HEMOLYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice-box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5(1-10)	.9	.1	.5	Add cells 1 hr.	.5	.5	-
.25(1-10)	1.15	.1	.5	37° C.	.5	.5	-

TABLE XXXV.

Positive normal serum and E. histolytica antigen obtained by saline extraction.

POSITIVE SERUM	NACL	E.HIST. SALINE EXT. ANTIGEN	COMPLEMENT 2 F.U.*		SHEEP CELLS 2%	HEMOLYSIN 2 F.U.	RESULTS
.1	1.4	0	.5	Ice box incubation	.5	.5	-
.1	1.3	.1	.5	18 hrs.	.5	.5	-
.5(1-10)	.9	.1	.5	Add cells 1 hr.	.5	.5	-
.25(1-10)	1.15	.1	.5	37° C	.5	.5	-

* A positive normal serum refers to a normal which will give a four plus fixation with Sherwood's alcoholic lipoid antigen III.

F.U. Full unit

\- No fixation.

TEST 3.

In this test, comparisons were made of the E. histolytica alcoholic lipoid antigen which had been used in the preceding tests and Sherwood's antigem III. The quantative technique devised by Sherwood was again used. Two normal sera were chosen, one which had formerly given a four plus fixation when set up by Sherwood, and one which had shown no fixation in a previous test. Both antigens were diluted according to the Sherwood technique: The alcoholic antigen was dropped into normal saline, caref ully shaking after each drop, making a one to five dilution. This was then made up with an equal volume of saline, making a final dilution of one to ten.

The following table XXXVI gives the results. The positive normal serum which had shown a four plus fixation in a previous test, again showed fixation with Sherwood's antigen III, while no fixation was demonstrated with the antigen which was prepared by the Heathman technique. The negative normal serum again showed no fixation with either antigen. This again points to the fixation of the complement as in some way connected with the concentration of the antigen.

TABLE XXXVI

A comparison of the two E. histolytica alcoholic lipoid antigens, one made by the Heathman , and the other by the Sherwood technique.

POSITIVE NORMAL SERUM*	HEATHMAN LIPOID ANTIGEN	NACL	COMPLEMENT 2 F.U.*		SHEEP CELLS 2 %	HEMO-LYSIN 2 F.U.	RESULTS
.1	0	1.4	.5		.5	.5	-
.1	.1	1.3	.5	Ice box	.5	.5	-
.5(1-10)	.1	0.9	.5	incubation	.5	.5	-
.25(1-10)	.1	1.15	.5	18 hrs.	.5	.5	-
				Add cells			

POSITIVE NORMAL SERUM	SHERWOOD LIPOID ANTIGEN	NACL	COMPLE-MENT 2 F.U.	incubate 1 hr 37° C.	SHEEP CELLS 2%	HEMO-LYSIN 2 F.U.	RESULTS
.1	0	1.4	.5		.5	.5	-
.1	.1	1.3	.5		.5	.5	4/
.5(1-10)	.1	0.9	.5		.5	.5	4/
.25(1-10)	.1	1.15	.5		.5	.5	4/

NEGATIVE NORMAL SERUM *	HEATHMAN LIPOID ANTIGEN	NACL	COMPLEMENT 2 F.U.	SHEEP CELLS 2%	HEMO-LYSIN 2 F.U.	RESULTS
.1	0	1.4	.5	.5	.5	-
.1	.1	1.3	55	.5	.5	-
.5(1-10)	.1	0.9	.5	.5	.5	-
.25(1-10)	.1	1.15	.5	.5	.5	-

NEGATIVE NORMAL SERUM	Sherwood LIPOID ANTIGEN	NACL	COMPLEMENT 2 F.U.	SHEEP CELLS 2%	HEMO-LYSIN 2 F.U.	RESULTS
.1	0	1.4	.5	.5	.5	-
.1	.1	1.3	.5	.5	.5	-
.5(1-10)	.1	0.9	.5	.5	.5	-
.25(1-10)	.1	1.15	.5	.5	.5	-

* Positive normal serum is a serum which shows four plus fixation with Sherwood's antigen III.
Negative normal is a normal serum which does not show fixation with Sherwood's antigen III.

F.U. Full unit
\- No fixation
4 / Four plus fixation.

TEST 4.

In this test a series of apparently normal medical students were bled and the sera set up with Sherwood's alcoholic antigen, using the quantative technique. The object was to find more of the normal type of serum which gave a four plus fixation with the alcoholic lipoid antigen. The results of this test , as given in Table XXXVII , show that all of the sera tested failed to fix complemetn. However an interesting phenomonon was shown in the rapidity of hemolysis in some of the tubes as compared with others. Some took as long as one andone half hours to show complete hemolysis, while others were hemolysed in fifteen minutes. Those which hemolysed slowly are indicated in Table XXXVII. The technique of the test is given in Table XXXVIII.

It is realized that since these tests were set up only once, a repetition of any of the immunolgical part of this work might disclose errors in technique.

TABLE XXXVII.

Complement-fixation using seventeen normal sera, with Sherwood's antigen III.

NAME	TUBE 1 0.1 SERUM	TUBE 2 0.5(1-10)	TUBE 3 .25(1-10)	FINAL RESULT
1. Capsey	- *	-	-	-
2 Love	-	-	-	-
3. Conklin	Slow- *	-	-	-
4 Oakes	Slow-	Slow-	Slow-	-
5. Pfeutze	-	-	-	-
6. True	-	-	-	-
7. Anderson	Slow-	Slow-	Slow-	-
8. Leger	-	-	-	-
9. Smith	Slow-	-	-	-
10 Schmidt	-	-	-	-
11. Haskell	-	-	-	-
12. Morine	-	Slow-	-	-
13. Fredeen	-	-	-	-
14 Pallette	Slow-	-	-	-
15 Griffen	-	-	-	-
16 Athy	-	-	-	-
17 Hubbard	-	-	-	-

* - No fixation

Slow- Slow hemolyzation

See Table XXXVIII for set up and controls.

TABLE XXXVIII.

The amounts of materials used and the general set-up for test 4.

(Results given in Table XXXVII.)

Student's Serum	Antigen III	Nacl	Complement 2 Full Units		Sheep cells 2%	Hemolysin 2 Full units
.1	0	1.4	.5	Ice-box	.5	.5
.1	.1	1.3	.5	incubation	.5	.5
.5(1-10)	.1	0.9	.5	18 hrs	.5	.5
.25(1-10)	.1	1.15	.5	Add cells	.5	.5
				1 hr. 37° C.		

Control 1. Tube one of each series acts as individual serum control. No fixation in any case occurred.

Control 2. .1 known positive serum
.1 Antigen III
1.3 Nacl
.5 Complement 2 Full units.
Following incubation in ice-box 18 hrs
.5 2% sheep cells
.5 Hemolysin 2 Full units.
Fixation in these tube occurred.

Control 3. .1 Known negative serum
.1 Antigen III.
1.3 Nacl
.5 Complement 2 Full units.
Following incubation in ice box 18 hours
.5 2% sheep cells
.5 Hemolysin 2 Full units.
No fixation in this tube occurred.

SUMMARY OF TESTS.

1. An anti serum may be prepared against E. histolytica by animal inoculation.
2. An antigen may be prepared by the method given by Heathman, which will fix complement in the presence of immune serum.
3. A more sensitive antigen may be prepared by concentration(Sherwood's alcoholic lipoid antigenIII)
4. The one rabbit used in inoculation of the antigen prepared by the Besredka, saline extraction method did not produce any demonstrable antibodies magainst B. hominis.
5. The Besredka saline extraction method produces an antigen inferior to the lipoid antigen obtained by alcoholic extraction.
6. Certain normal sera show a tendency tword inhibition of the hemolytic system.
7. No cross reaction was discernible between E. histolytica and B. hominis in the complement fixation reaction.

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